THE DISCOCYTE–ECHINOCYTE TRANSFORMATION AS AN INDEX OF HUMAN RED CELL TRAUMA[1]

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Abstract. Scanning electron microscopic observation of blood samples before, during and after cardiopulmonary bypass during cardiovascular surgery revealed that 4 to 25% of the red blood cells undergo a progressive transformation of discocyte to echinocyte. A morphological index, I, was developed and the change in I (Ir) was found to correlate well with measurements of free plasma hemoglobin. Incubation of blood samples form normal subjects for 90 minutes at 37°C resulted in no increase in Ir whereas incubated samples from patients following cardiopulmonary bypass showed an increased Ir to a mean value of 1140 ± 185. Incubation therefore appears to uncover sublethal red cell damage caused by extracorporeal circulation. It is suggested that this technique is a sensitive index of red cell trauma which may have useful clinical applications.

OHIO J. SCI. 76(5): 225, 1976

Appreciation of the fine details of red blood cell shape can provide information of great importance; yet the various shapes that the red cell can assume are just beginning to be analyzed in a critical fashion. Until recently, red cell shape could only be studied with the optical microscope, i.e. with a limiting resolution of 0.2 micron. The situation changed with the advent of the scanning electron microscope, and we are now able to see the details of cell shape with a tenfold improvement in resolution and a depth of field that permits an appreciation of the three-dimensional shape of the cells (Salsbury and Clarke, 1967). It is the purpose of this communication to demonstrate how morphologic changes can be quantitated and used as an index of red cell trauma.

MATERIALS AND METHODS

Studies were carried out with the aid of Mini-Rapid Scan, ISI, scanning electron microscopy (SEM) on fifteen patients undergoing cardiovascular surgery. Small quantities of blood were obtained from: banked donor blood; the patients before cardiopulmonary bypass; the arterial side of the extracorporeal circuit at 15, 30 and 60 minutes of bypass; and the patients two hours post-operatively. Two or three drops of a blood sample were allowed to fall initially into 10 ml of a 0.1% gluteraldehyde-sucrose-buffer solution (pH—7.40, 520 mOsM) for at least 30 minutes. The cells were centrifuged and resuspended in 10 ml of 3% gluteraldehyde solution where they remained for at least 60 minutes to complete fixation. Following fixation the cells were washed with Isoton (Coulter Electronics, Inc.) in order to remove any protein, salt crystals or other material that might have been deposited on the cell surface. Dehydration was accomplished by passing the cells successively through 65, 80, 90, 95 and 100% concentrations of ethyl alcohol followed by propylene oxide. A small drop of the final cell suspension in propylene oxide was then allowed to spread on a metal stub; dessication took place almost instantaneously. The cells were coated with Gold-Palladium in a vacuum evaporator to make the surface conductive for observation in the SEM.

Two hundred to 400 cells of each sample were counted at a magnification of 5000× and were classified into four types following the system of nomenclature of Bessis (1974). Since discocytes undergo a progressive transformation with the echinocyte type III representing the most severe damage (fig. 1), a morphological index was calculated by arbitrarily assigning a weighting factor to each cell type as follows: discocyte, 0; echinocyte type I, 1; echinocyte type II, 2; echinocyte type III, 3.

The average number of each cell type per 100 cells in the sample was multiplied by its weighting factor. The sum of these products equals the morphologic index, I, of that sample. Thus a sample with all discocyte forms would have an index, I, of 0 whereas a sample in which all cells were of echinocyte type III form would have I=300.

Comparison of the change in the number of

[1]Manuscript received July 8, 1975 and in revised form May 31, 1976 (#75-45).

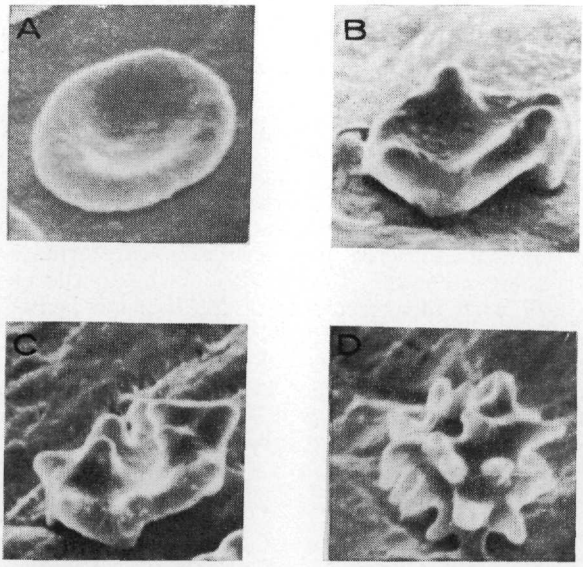

FIGURE 1. Scanning electron micrographs of discocyte (A), echinocyte type I (B), echinocyte type II (C), and echinocyte type III (D).

distorted cells in any one patient was made by calculating the percent change from the control

$$\frac{Iex-IC}{IC}\times 100\%$$

and is reported as the relative morphologic index, Ir. In the special case where IC=0, Ir=Iex×100. The extent of hemolysis during cardiopulmonary bypass was determined and expressed as mg% free plasma hemoglobin (Brodine and Vertrees, 1964).

To determine red cell morphology following incubation, six blood samples from four normal subjects (twice from two individuals) were collected in heparin and incubated under sterile conditions at 37°C for up to 24 hours. Samples were removed each hour, fixed by the above protocol and prepared for SEM observation. Blood samples obtained from patients before bypass, from the bypass circuit, from the patients two hours-post-operatively, and samples of banked donor blood were incubated under the same conditions for up to 90 minutes. Samples were removed every 30 minutes and indexed by SEM observation.

To examine the possibility that chemical changes occur in the plasma of blood exposed to damage in the extracorporeal circuit, blood samples from banked donor blood, and from the bypass after 30 and 60 minutes were centrifuged at 1,000×G for 10 minutes. The supernatant plasma was removed and the cells were resuspended in plasma obtained from normal individuals and from the patients before bypass. Cells from samples drawn before bypass were similarly resuspended in the 30 and 60 minute bypass plasma. After 90 minutes of incubation at 37°C, samples were prepared for SEM observation by the methods outlined above.

RESULTS

In the present study it was observed that during cardiopulmonary bypass 4% to 25% of the erythrocytes undergo a detectable steady-state morphologic change of discocyte to echinocyte (fig. 2). When quantitated by means of the morphological index (I) it was found that I was 0 in normal subjects, ranged from 0 to 9 in patients prior to cardiac surgery, and from 4 to 42 in patients after 60 minutes of bypass (fig. 3A).

Because of the wide variation among individual patients and the correlation of pre-bypass levels of echinocytes with the extent of change observed during bypass, the morphological change was expressed as a percent increase using the relative morphologic index Ir. It was found that Ir increased to 365±85 while the patient is on cardiopulmonary bypass (fig. 3B). There also appears to be a correlation between I and the extent of hemolysis of red cells as measured by free plasma hemoglobin determinations in patients during the bypass procedure. Patients with low hemolysis levels had small values for I and conversely high hemolysis levels were correlated with large

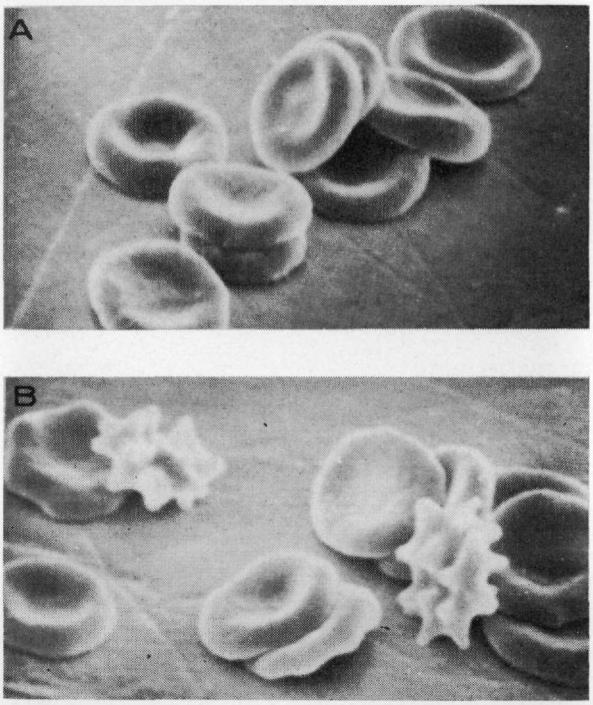

FIGURE 2. Scanning electron micrographs of RBCs before cardiopulmonary bypass (A), and after 60 minutes of bypass (B).

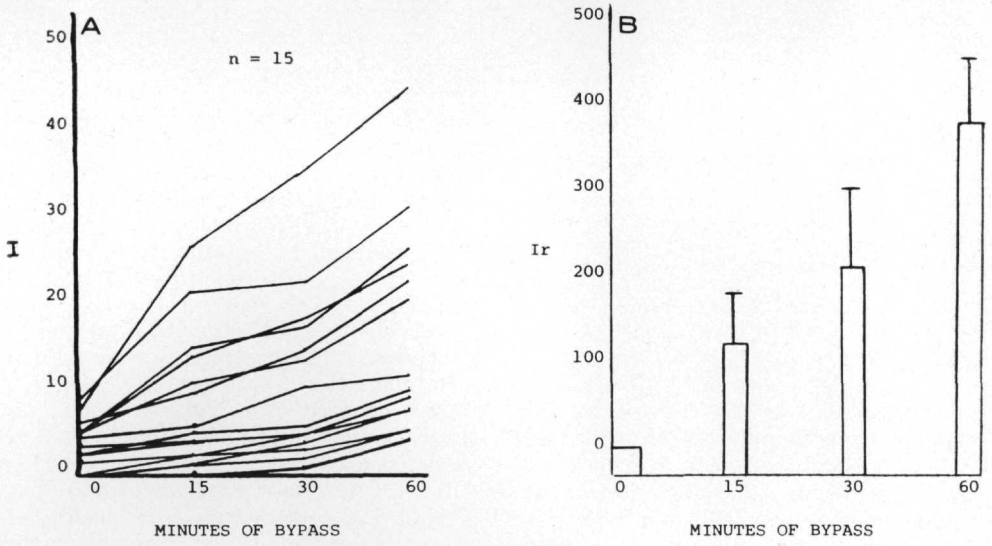

FIGURE 3A. Morphological index (I) during 60 minutes of cardiopulmonary bypass in 15 patients undergoing cardiac surgery.

3B. Relative change (Ir) in the morphological index (I) of distorted cells observed during cardiopulmonary byoass. Bars represent mean ± SD.

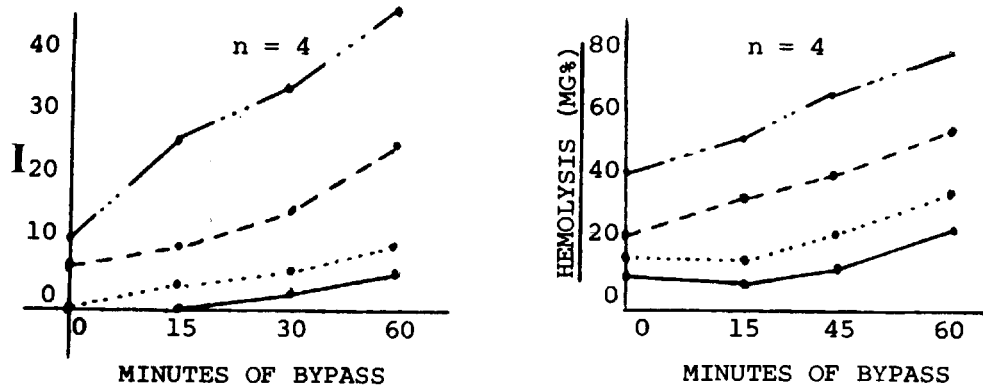

FIGURE 4. Changes in morphologic index (I) and free plasma hemoglobin during cardiopulmonary bypass for 4 of the 15 patients (left). Similar correlation of I and hemolysis was observed in 13 of the 15 patients studied (right).

values of I during bypass (correlation coefficient = 0.888 for 14 patients) (fig. 4).

It was determined that incubation of blood samples at 37° C resulted in a time dependent increase in Ir. Figure 5 indicates the extent of the morphological change following 90 minutes of incubation at 37° C. Red blood cells from normal individuals began to show detectable morphologic changes when incubated for 6 hours whereas there was no detectable change at 90 minutes (table 1). Incubation of pre-bypass samples from patients with cardiovascular disease and blood samples from the bypass circuit, however, demonstrated an increased number of echinocytes (Ir = 280 ± 65 and 1140 ± 185, respectively) following only 90 minutes of incubation. The majority of distorted cells produced by 90 minutes of incubation were echinocytes type II and III (table 2).

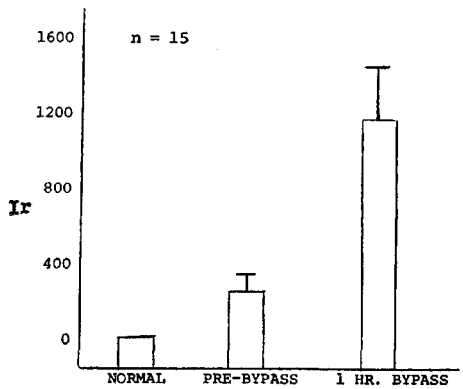

FIGURE 5. Increase in the number of echinocytes, expressed as relative morphological index, Ir (mean ± SD) following 90 minutes of sample incubation at 37°C. Each bar represents the change observed compared with the pre-incubation value.

TABLE 1

Echinocyte Morphological Index in blood samples from normal subjects following incubation at 37° C for 1 to 24 hours.

Incubation time, hr.	Morphological Index, (I) Subject Number 1	2	3	4	5	6
1	0	1	0	0	1	0
2	0	0	0	0	1	0
3	0	0	0	1	0	0
4	0	0	0	0	0	0
6	1	2	1	1	0	2
10	10	16	21	12	8	14
12	13	20	27	16	20	20
24	68	78	83	64	73	93

Resuspension of red cells, obtained before bypass, in post-bypass plasma, without incubation, resulted in an increase in the relative morphological index (Ir) to 380 ± 190 and incubation of the resuspension further extended Ir to 1220 ± 210 (fig. 6). However, resuspension of cells obtained after bypass in plasma obtained before bypass or from normal individuals failed to reverse the echinocytes to the biconcave disc configuration.

Values of I in banked donor blood

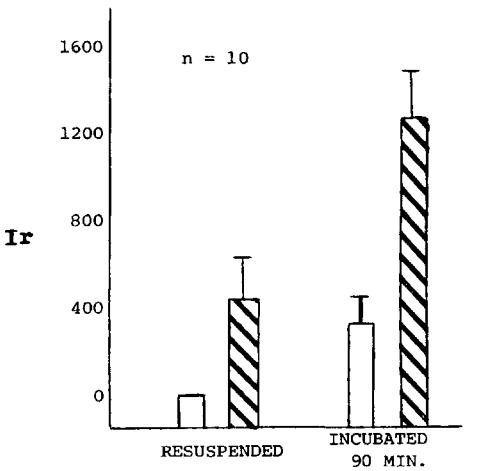

FIGURE 6. Effect of post-bypass plasma on red cells obtained from the patient prior to cardiopulmonary bypass. Each bar represents the change in morphological index compared with the control before resuspension or incubation. Pre-bypass RBC in pre-bypass plasma-open bars; pre-bypass RBC in post-bypass plasma-diagnonal lined bars.

ranged from 60 to 100 primarily due to the large proportion of echinocytes type III in the samples. These echinocytes, however, displayed a tendency to revert to the biconcave disc form on incubation or when resuspended in normal plasma.

Of particular interest was the fact that the number of echinocytes decline after the bypass is discontinued. At two hours post-operatively the value of I decreased to one-half of that found after 60 minutes of bypass.

DISCUSSION

Experimentally manipulated red cells and red cells of patients with certain diseases can have steady-state configurations other than the biconcave disc (Weinstein, 1974). In the present investigation scanning electron microscopy of red cells after cardiopulmonary bypass revealed that some of the cells assume alternative shapes, irregularly contoured discocytes (fig. 1A), flat red cells with spicules (fig. 1B), and a few cells ultimately transform into spherical cells which bear 30 to 40 uniformly spaced projections or "spicules" over their surface (fig. 1D). These spiculated forms have been given the name of echinocyte, which refers to their superficial resemblance to echinoderms, such as sea urchins (Bessis and Lessin, 1970). The initial cause of the transformation presumably results from physical or chemical damage of the red blood cells. Forces of possible importance in this phenomenon during cardiopulmonary bypass include shearing stress, turbulence, pressure fluctuations, crushing, interaction of erythrocytes with

TABLE 2

Distribution of echinocytes by type and the morphological index (I) following incubation at 37°C for 90 minutes in blood samples obtained before and after cardiopulmonary bypass.

Patient	Pre-bypass				Post-bypass			
	No. of echinocytes			I	No. of echinocytes			I
	Type I	Type II	Type III		Type I	Type II	Type III	
1	4	1	0	6	21	5	4	43
2	5	0	0	5	23	25	9	100
3	3	0	0	3	27	6	2	45
4	10	1	1	15	18	21	5	75
5	6	0	0	6	11	53	21	180
6	6	2	0	10	6	57	25	195
7	1	0	0	1	23	4	1	34
8	5	3	1	14	4	40	50	234
9	16	6	2	31	3	55	26	191
10	8	5	0	18	4	22	73	267
11	7	7	1	24	2	40	51	235
12	8	4	0	16	1	10	82	267
13	2	0	0	2	47	9	3	74
14	14	9	2	38	1	8	90	287
15	14	11	1	37	1	13	85	282

nonendothelial surfaces of the bypass circuitry, and chemical changes which may occur in the plasma of blood exposed to damage in the extracorporeal circuit.

Although the discoid shape of red blood cells permits them to accomodate to the extreme deformations required when the cells traverse capillaries with diameters smaller than their own, it is not known whether the spicule formation induced by extracorporeal circulation, *per se*, has major consequences on red cell survival *in vivo* or causes dramatic alterations in the viscosity or flow properties of the blood. Investigators have reported, however, that some factors inducing the discocyte-echinocyte transformation lead to measurable changes in the mechanical properties of the surface of the red cell (Leblond, 1973).

The *in vivo* fate of echinocytes from banked donor blood is uncertain. Our preliminary experiments and reports of others (Mollison, 1967; Bessis, 1974) suggest, however, that these echinocytes are either immediately removed or revert to normal when they re-enter the body. Further investigation will be necessary to determine whether a distinction must be made between the echinocytes from banked donor blood and those produced by damage in the extracorporeal circuit since echinocytes from banked donor blood revert to biconcave discs whereas bypass samples show increased numbers of echinocytes on incubation.

Since Ir is increased in samples obtained from patients before and during bypass in a much shorter time of incubation than in blood obtained from normal individuals, it is suggested that sublethal damage to the complex mechanisms which govern the structure of the cell membrane may be exposed by the incubation.

It was observed that some patients undergoing cardiopulmonary bypass surgery had some echinocytes in the blood prior to bypass. One explanation for this may be the fact that the inner surfaces of the heart and blood vessels in patients with heart disease are usually rougher than those found in normal individuals. As the red cells encounter the vessel walls and collide with roughened cardiovscular surfaces, damage could be produced which may account for the increased number of distorted cells in control and incubated samples compared with those found in normal subjects.

The study of the discocyte-echinocyte transformation is therefore proposed as an assay for the extent of red blood cell trauma during extracorporeal circulation which may be useful in the investigation of sources of damage and the evaluation of methods for the prevention of this potentially deleterious event. The demonstration that incubation of sublethally damaged red cells leads to an increase in Ir in a very much shorter time than with normal cells increases the sensitivity of the method. It is suggested that this assay may also be a good index of the severity of vascular lesions which might be useful in the clinical evaluation of patients with heart and blood vessel disease.

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